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Recovery of Ins(1,4,5)-trisphosphate-dependent calcium signaling in neonatal gonadotrophs

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Abstract

Pituitary gonadotrophs express non-desensitizing gonadotropin-releasing hormone (GnRH) receptors and their activations leads to inositol 1,4,5-trisphosphate ($InsP_3$)-dependent Ca^{2+} mobilization. When added in physiological concentration range GnRH induces baseline Ca^{2+} oscillations, whereas in higher concentrations it induces a prolonged spike response accompanied with non-oscillatory or oscillatory plateau response. Here, we studied the recovery of calcium signaling during repetitive stimulation with short (10–30 s) GnRH pulses and variable interpulse intervals in neonatal gonadotrophs perfused with Ca^{2+}/Na^+-containing, Ca^{2+}-deficient/Na^+-containing, and Ca^{2+}-containing/Na^+-deficient media. In Ca^{2+}/Na^+-containing medium, baseline Ca^{2+} oscillations recovered without refractory period and with a time constant of ∼20 s, whereas the recovery of spike response occurred after 25–35 s refractory period and with a time constant of ∼30 s. During repetitive GnRH stimulation, removal of Ca^{2+} had only a minor effect on baseline oscillations but abolished spike response, whereas removal of Na^+ slightly extended duration of baseline oscillations and considerably prolonged spike response. These results indicate that two calcium handling mechanisms are operative in gonadotrophs: redistribution of calcium within $InsP_3$-sensitive and -insensitive pools and a sodium-dependent calcium efflux followed by calcium influx. Redistribution of Ca^{2+} within the cell leads to rapid recovery of $InsP_3$-dependent pool, whereas the Na^+-dependent Ca^{2+} efflux pathway is activated by spike response and limits the time of exposure to elevated cytosolic Ca^{2+} concentrations.

1. Introduction

Differentiation of pituitary secretory cells arises during embryonic life. In rats this process occurs between 15 and 18 days of embryonic age [1]. In newborn animals, the neonatal population of gonadotrophs is gradually substituted with the postnatal generation of cells during pre- and peripubertal period. Both types of gonadotrophs express G protein-coupled gonadotropin-releasing hormone (GnRH) receptor [2,3] and their activation leads to increase in inositol 1,4,5-trisphosphate ($InsP_3$) production and release of calcium through $InsP_3$ receptor-channel expressed in the endoplasmic reticulum membrane [4–6]. On the other hand, neonatal but not postnatal gonadotrophs express melatonin MT1 receptor [7], which inhibits GnRH-induced signaling and secretion [8].

In contrast to postnatal gonadotrophs, the intracellular signaling by GnRH in neonatal gonadotrophs has been incompletely characterized. In general, GnRH-induced calcium signals differ in the shape, frequency, and amplitude. Low to intermediate GnRH concentrations induce periodic calcium release with the $[Ca^{2+}]_i$ oscillating around its resting level, with a cycle frequency of 10–30 min^{-1}. These high-frequency baseline calcium oscillations are not unique for gonadotrophs, but were also observed in other cell types [9,10]. Higher GnRH doses induce biphasic response composed of an initial large non-oscillatory spike response and sustained baseline oscillations [11–16]. Both patterns of calcium signals are able to stimulate hormone secretion via exocytosis, as assessed by measurements of capacitance [17]. In neonatal gonadotrophs GnRH also generates an initial spike response not accompanied with sustained oscillations [6], and such monophasic pattern of signaling is comparable to that observed in GnRH-stimulated immortalized αT3-1 neonatal gonadotrophs [18]. The dual control of $InsP_3$ receptor-channels, by $InsP_3$ and calcium [19,20],

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provides a satisfactory explanation for the transition from baseline oscillations to biphasic and monophasic responses [21]. Low-frequency baseline oscillations with no changes in the frequency in response to increasing concentration of GnRH were also observed in neonatal gonadotrophs [6]. These oscillations resembled the thapsigargin-stimulated oscillations observed in postnatal gonadotrophs [22]. Thapsigargin is a blocker of SERCA type calcium ATPase [23], and these oscillations in neonatal gonadotrophs may reflect a decrease in capacity of this pump to reload calcium during the prolonged recording and reduced content of calcium in intracellular stores.

Extracellular calcium is not essential to initiate calcium oscillations in neonatal gonadotrophs [6]. On the other hand, the role of calcium influx in sustained and repetitive calcium signaling of neonatal gonadotrophs was not studied previously. Furthermore, the mechanism of calcium efflux and the kinetics of recovery of signaling during repetitive agonist stimulation have been incompletely characterized in these cells. Here, we addressed these questions by studying GnRH-induced calcium signaling in cells bathed in normal ion solution, as well as in Ca^{2+}-deficient and sodium-deficient medium. The focus in study was on the relevance of signaling pattern on calcium efflux through the plasma membrane and calcium redistribution within the cells, as well as on the impact of these two processes on recovery of agonist-induced calcium signals. The nystatin-perforated patch-clamp recording of apamin-sensitive Ca^{2+}-dependent K^+ currents was used to monitor GnRH-induced $[Ca^{2+}]_i$ changes during repetitive stimulation with short GnRH pulses. This was done to avoid the buffering effects of calcium dyes on the pattern of signaling [5] and to get better estimate of sub-plasma membrane calcium, an important issue in studies on the impact of calcium signaling pattern on its efflux through plasma membrane pathways. The results indicate that the pattern of agonist-induced calcium signaling determines which process, redistribution within the cell or calcium removal from the cell, dominates during agonist-induced calcium signaling. This in turn affects the kinetics of recovery of signaling and the source of calcium during the replenishment of Ca^{2+} stores.

2. Materials and methods

2.1. Cell preparation and culture

Neonatal rat pituitary cells were prepared from rat pups kept with their mothers under a controlled 12-h light/dark cycle (with lights on at 6.00 a.m.) from the age of 2 days. Six- to 10-day-old pups were killed by decapitation between 8.30 and 10.00 a.m. The anterior pituitary glands were rapidly removed under sterile conditions, gently disrupted and dissociated with papain [24]. Dispersed cells were separated from the cell debris and cell clusters using a discontinuous albumin gradient and then resuspended in Eagle's minimal essential medium (MEM) [25]. Dissociated cells were seeded on 35-mm culture dishes covered with poly-L-lysine and cultured in MEM, supplemented with 5% neonatal rat serum from the pituitary donors + 5% fetal calf serum in air/CO_2 at 37 °C. Cultures were used for experiments within 48 h after dissection. All experiments were carried out in accordance with the European Communities' Council Directives (86/609/EEC) and with approval of the Institutional Animal Care and Use Committee.

2.2. Current recordings

Nystatin-perforated patch-clamp recording of Ca^{2+}-activated potassium current through apamin-sensitive channels was used to monitor GnRH (Sigma)-induced $[Ca^{2+}]_i$ changes [4,5,26]. GnRH-induced currents were recorded using Axopatch-1D amplifier (Axon Instruments, Union City, CA, USA). Thin-walled borosilicate glass tubes of 1.65 mm external diameter (Kavalier, Czech Republic) were pulled on the horizontal Flaming Brown P-97 model puller (Sutter Instruments, Novato, CA, USA). The tip of the pipette had an outer diameter of about 3 μm and the pipette resistance was 4–10 MΩ. Cells were investigated 10 min after seal formation when the perforated seal resistance was 20–50 MΩ. No series resistance compensation was used. The plasma membrane potential was kept at −40 mV to increase the amplitude of Ca^{2+}-activated potassium current, for which the measured equilibrium potential was about −90 mV. At −40 mV, the voltage-gated Ca^{2+} channels in postnatal gonadotrophs are half inactivated [4], but there was no significant effect of nifedipine, a blocker of these channels, on the amplitude of GnRH-induced Ca^{2+}-activated potassium current in neonatal gonadotrophs [6], indicating that background Ca^{2+} conductance does not substantially contribute to the electrophysiologically monitored $[Ca^{2+}]_i$ changes. Correction for the Donnan potential due to the absence of nonpermeable anions in the pipette solution (see below) was omitted. Whole-cell currents were stored in digital forms and analyzed using the Digidata 1200A interface and pClamp 8 software package (Axon Instruments). Signals were filtered at 1 kHz and sampled at 2 kHz. Experiments were carried out at 20–25 °C.

2.3. Solutions

During the patch-clamp recording, dishes with cell cultures were continuously perfused with extracellular solution containing (in mM): 160 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES); pH adjusted to 7.3 with 1 M NaOH. In some experiments *N*-methyl-D-glucamine (NMDG) was substituted for sodium chloride. Junction potential in Na^+-deficient solution (8 mV) was corrected. Patch electrodes used for perforated patch-clamp recordings were filled with an intracellular solution containing (in mM): 140 KCl, 1 $MgCl_2$, and 10 HEPES; pH adjusted with 1 M KOH to 7.2. Nystatin (Sigma) and dispersing agent

Pluronic F-127 (Molecular Probes) were added to the intracellular solution from stock solutions in dimethylsulfoxide to obtain a final concentration of 250 and 500 μg/ml, respectively. In some experiments, the whole-cell recording was performed and 2 mM ATP, 0.3 mM GTP, and 10 μM $InsP_3$ (Sigma) were added to the intracellular solution. The osmolarity of the internal solutions was 283–287 mOsm.

2.4. Drug application

The drug-containing and drug-free solutions were applied with a fast gravity driven perfusion system consisting of an array of 10 glass tubes each approximately 400 μm in diameter. Movement of the glass tube array and solution application was controlled by a step motor and miniature Teflon solenoid valves operated by a microcomputer. The application array was routinely positioned ∼200 μm distance from the recorded cell and ∼50 μm above the surface of the culture. Complete exchange of the solution around recorded cells required <50 ms, as estimated from altered K^+ current (10–90% rise time, not shown). During the recording from a single cell, GnRH was repeatedly applied at 10- to 120-s intervals to study recovery of GnRH-induced Ca^{2+} responses.

2.5. Data analysis

The amplitude of the Ca^{2+}-dependent K^+ currents, frequency and duration of current transients, and the latency, i.e. the time to onset of the current induced by GnRH, were analyzed using pClamp 8 software (Axon Instruments). Recovery data for GnRH-induced currents were curve-fitted to an equation for a single exponential curve using Sigma Plot 3.0 software. This program was also used for evaluating statistical significances. All values are reported as means ± S.E.M. The differences between means were tested by Student's *t*-test, and $P < 0.05$ were considered as significant.

3. Results

3.1. Patterns of GnRH-induced calcium signaling in neonatal gonadotrophs

The majority of neonatal gonadotrophs stimulated with GnRH responded with baseline oscillations, which frequency (ranging from 12 to 38 min^{-1}) depended on agonist concentration. The averaged duration of single transients in cells exhibiting baseline oscillations was 2.60 ± 0.07 s, and we termed this type of signaling *rapid baseline calcium oscillations*. Cells exhibiting this type of signaling regularly responded with changes in the pattern of signaling from baseline oscillations to a prolonged spike response when stimulated with higher GnRH doses. There were two types of spike responses: one was sustained with plateau baseline oscillations, and we termed this type of signaling *biphasic calcium response*, and the other was sustained with non-oscillatory plateau signals, and we termed this type of signaling *monophasic calcium response*. In a fraction of neonatal gonadotrophs, GnRH also stimulated *slow baseline oscillations*, which frequency ranged from 3 to 12 min^{-1} and was independent of agonist concentration. We termed these oscillations slow because the duration of individual transients in such oscillating cells was three to four times longer compared to individual spikes in cells exhibiting rapid baseline oscillations, and there was no transition from oscillatory to biphasic or monophasic signaling pattern at higher GnRH concentration.

When stimulated with 10 nM GnRH, about 20% of cells (57 out of 275) responded with baseline oscillations, which frequency was 17.6 ± 0.7 min^{-1} (Fig. 1A). In our experimental conditions, these oscillations started 5–9 s after beginning of GnRH application (latency) and maximum amplitude of current was reached within 15 s. In the presence of agonist, the amplitude of oscillations declined with a half-decay time of 16.3 ± 5.2 s to an oscillatory plateau level. Almost 50% of cells (130 out of 275) stimulated with 10 nM GnRH responded with biphasic calcium response (Fig. 1B). The averaged frequency of sustained oscillations was 23.8 ± 1.6 min^{-1}, the amplitude of initial calcium spike was 57.9 ± 5.2 pA and the amplitude of oscillatory plateau was 13.8 ± 0.9 pA. In 23% of cells (63 out of 275), 10 nM GnRH induced only monophasic response (Fig. 1C). The peak amplitudes of monophasic signals was 60.2 ± 4.1 pA that was highly comparable with the initial spikes in cells

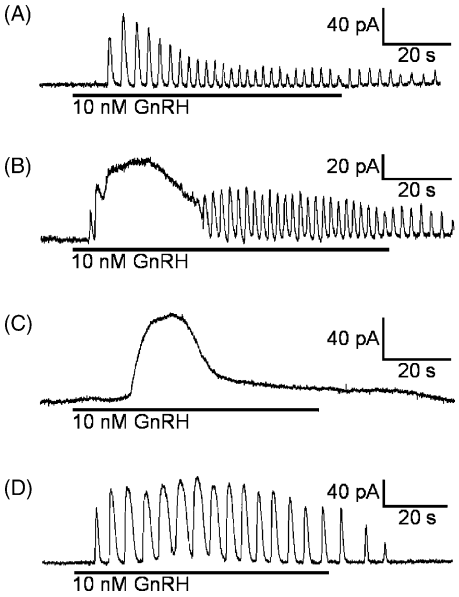

Fig. 1. Patterns of GnRH-induced calcium signals in neonatal gonadotrophs. (A) Rapid baseline oscillations; (B) biphasic response; (C) monophasic response; (D) slow baseline oscillations. In this and following figures, apamin-sensitive calcium-activated potassium current was used as an indicator of changes in intracellular calcium concentrations and cells were voltage-clamped to −40 mV. All experiments were done in 24- to 48-h-old cultures of pituitary cells from neonatal animals (6–10 days old animals). Horizontal lines indicate duration of perfusion with 10 nM GnRH.

responding with biphasic signals. The latency period in cells responding with biphasic and monophasic response ranged form 5 to 20 s. In the residual cells (25 out of 275), 10 nM GnRH induced slow baseline oscillations with amplitude usually higher than that of high-frequency baseline oscillations (102.0 ± 13.7 pA; Fig. 1D).

3.2. Recovery of rapid baseline calcium oscillations

To study the recovery of rapid baseline calcium signaling during repetitive stimulation with GnRH, cells were stimulated with this agonist for 10 s, followed by the additional pulses at variable interpulse interval. In all cells initially responding to GnRH with this type of signaling, several subsequent applications of GnRH also induced rapid baseline oscillations. As shown in Fig. 2A–D, there was no obvious change in the frequency of oscillations during repetitive stimulation, whereas the amplitude of subsequent calcium signals was critically dependent on the interpulse interval. If a second GnRH pulse was elicited 10 s after the first, the peak amplitude of calcium transients were only about 40% of that observed during initial stimulation (not shown). Prolongation of the washing interval between two GnRH stimuli to 20 s (Fig. 2A) and 30 s (Fig. 2B) increased the amplitude of the second response, and the majority of baseline oscillatory responses fully recovered after 40–80 s of washing (Fig. 2C). The calculated recovery time constant of GnRH-induced rapid baseline oscillation was 20.8 ± 3.2 s ($n = 4$; Fig. 2E). The pattern of calcium signaling was highly reproducible during the repetitive stimulation of equal interpulse intervals in a single cell (Fig. 2C).

The recovery of GnRH-induced rapid baseline oscillations was also observed in cells bathed in Ca^{2+}-deficient medium (free calcium about 100 nM). As shown in Fig. 2D, the initial response in cells perfused with calcium-deficient medium was comparable to that observed in cells perfused in calcium-containing medium. The second and the third subsequent GnRH pulse applied in Ca^{2+}-deficient medium with the interval of 40 s induced the same pattern of response, but with amplitudes lower by about 20–30% compared to the first pulse. When calcium was added to the medium, the full recovery was reached (Fig. 2D, the fourth stimulus). These results indicate that removal of external calcium for a short time had no significant effect on the rate of recovery and the amplitude of GnRH-induced $[Ca^{2+}]_i$ baseline oscillatory response. Because GnRH receptor does not desensitize, these results also indicate that intracellular calcium stores responsible for baseline oscillations are quickly refilled in the absence of extracellular calcium, probably by recycling of calcium within the intracellular calcium pools.

3.3. Recovery of biphasic and monophasic calcium responses

In further studies, we estimated the recovery time of responses to variable GnRH concentrations in single cells. The

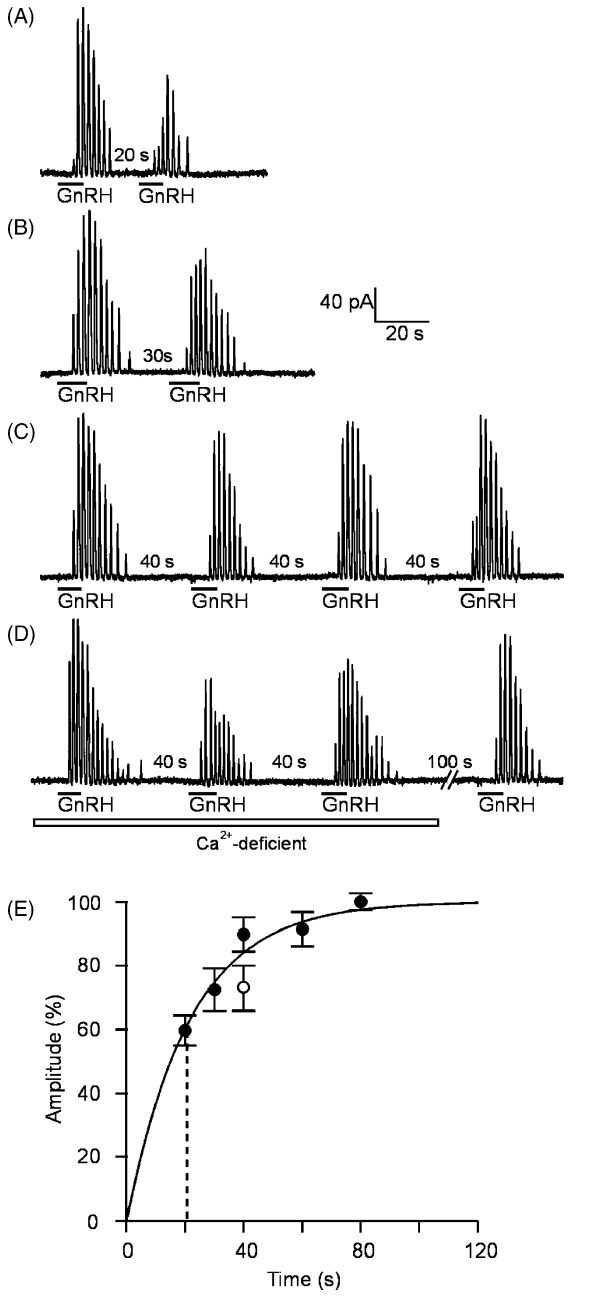

Fig. 2. Recovery of rapid baseline calcium oscillations during repetitive GnRH stimulation. (A–C) Effects of variable interpulse intervals on recovery of calcium signals. (D) Effects of removal of extracellular calcium on recovery of calcium signals. Horizontal lines indicate duration of GnRH application and horizontal bar indicates duration of perfusion with calcium-deficient medium. The current traces shown in panels A to D are from the same cell. Gonadotrophs were stimulated with 10 nM GnRH. (E) The amplitude of the second GnRH response expressed as percentage of control GnRH-induced current and plotted as a function of time interval between two GnRH pulses. Data from individual cells were fitted to a single exponential equation and a theoretical curve was drawn using the mean value of $\tau = 21.1 \pm 5.2$ s. Dotted vertical line indicates the calculated τ value. Each point represents the mean ± S.E.M. from four cells recorded in calcium-containing (filled circles) and calcium-deficient solution (open circle).

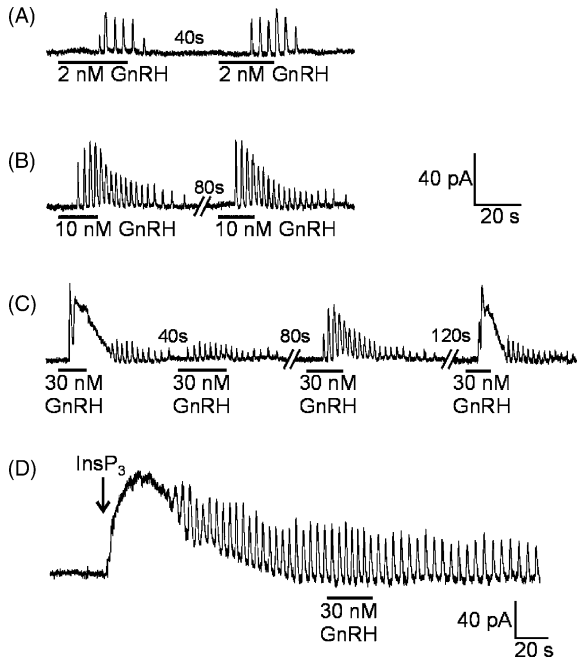

Fig. 3. Dependence of recovery of calcium signaling on GnRH concentration. (A–C) Recovery of response during repetitive GnRH stimulation in a single cell. Notice that 2 nM GnRH (A) and 10 nM GnRH (B) induced baseline oscillations, whereas 30 nM GnRH (C) generated biphasic response, as well as that the recovery time for three concentrations of GnRH was variable. (D) The lack of effects of GnRH (30 nM) on calcium signaling in cells with ongoing biphasic response induced by intracellular injection of 10 μM $InsP_3$. The $InsP_3$ was present in the intrapipette solution and was introduced into the cell during breaking through to the whole-cell configuration.

baseline oscillations induced by 2 nM GnRH recovered completely within 40 s (Fig. 3A), in contrast to cells stimulated with 10 nM GnRH that required 80 s (Fig. 3B). On the other hand, biphasic responses stimulated by 30 nM GnRH in the same cell recovered more slowly and in two steps. If the second stimulation with 30 nM GnRH was applied 40–80 s after the first application, only the sustained baseline oscillations recovered, whereas the full recovery of spike response required 100–120 s of washing (Fig. 3C). These results indicate that recovery of biphasic responses requires longer time compared to rapid baseline oscillations.

Next, we examined the status of agonist-sensitive calcium pool in activated cells. To do this, we initiated calcium signaling by injection of 10 μM $InsP_3$. In general, intracellular IP_3 application induced baseline oscillations, biphasic response, or monophasic response, but never slow baseline oscillations. As shown in Fig. 3D, the pattern of $InsP_3$-induced biphasic calcium signal was highly comparable to that observed in 30 nM GnRH-treated cells. Because of continuous diffusion of $InsP_3$ from the pipette, however, the sustained plateau oscillations lasted for a prolonged period. GnRH (30 nM) applied during ongoing $[Ca^{2+}]_i$ oscillations failed to induce a spike response in $InsP_3$-injected cells (Fig. 3D), in contrast to control cells (Fig. 3C), and only slightly increased the frequency of baseline oscillations in some cells (not shown). These results indicate that the agonist-sensitive calcium pool is depleted by initial spike response.

To analyze in more details the time of recovery of spike response, in further studies we selected cells that responded to GnRH with monophasic response, i.e. cells that do not show sustained baseline oscillations after the initial spike response. In these experiments, we used the same protocol as for rapid baseline oscillations, but longer (20–30 s) GnRH stimuli were applied to account for longer latency. In calcium-containing extracellular solution, no response could be elicited when the second GnRH pulse was applied within 30 s after the first stimulation (not shown). After this initial refractory period, GnRH-induced responses rapidly recovered (Fig. 4A–C) with $\tau = 28.5 \pm 4.1$ s ($n = 5$; Fig. 4E). Full recovery of the peak amplitude of non-oscillatory GnRH-induced response was observed when the washing interval between two GnRH pulses was about 100 s.

In the absence of external calcium, the first GnRH pulse generated response the pattern of which was comparable to that observed in cells bathed in Ca^{2+}-containing medium (Fig. 4D, first peak versus Fig. 4C, first peak). However, the second GnRH application induced substantially smaller response, and each subsequent GnRH applications in calcium-deficient medium failed to induce any $[Ca^{2+}]_i$ response. After re-addition of calcium to the medium, GnRH-induced $[Ca^{2+}]_i$ responses started to recover, and within 100 s the recovery reached 60–90% of control value (Fig. 4D). These results indicate the dependence of recovery time on the pattern of agonist-induced calcium mobilization and on the presence of calcium in perfusion medium. Whereas the rapid baseline calcium oscillations recover relatively fast and predominantly in extracellular calcium-independent manner, biphasic and monophasic release of calcium from intracellular pool leads to a delay in recovery time, which is probably needed for cells to reload agonist-sensitive intracellular calcium pool by calcium influx.

3.4. Recovery of slow baseline oscillations

In further studies, we used cells exhibiting slow baseline oscillations to test the hypothesis that duration of calcium transients represents the factor that determines whether calcium is predominantly relocated within the cells, as observed in rapid baseline oscillations, or removed from cells by the plasma membrane efflux mechanism, as shown in cells responding with monophasic/biphasic calcium signals. Slow baseline oscillations were often observed after repetitive stimulation (usually after 30 min of recording) in the same cell that exhibited rapid baseline oscillations (Fig. 5). When bathed in calcium-deficient medium, these cells responded to the first GnRH pulse with comparable amplitude and duration of spikes to that observed in cells perfused with calcium-containing medium (Fig. 6A versus Fig. 6B). However, in contrast to rapid baseline oscillations, the

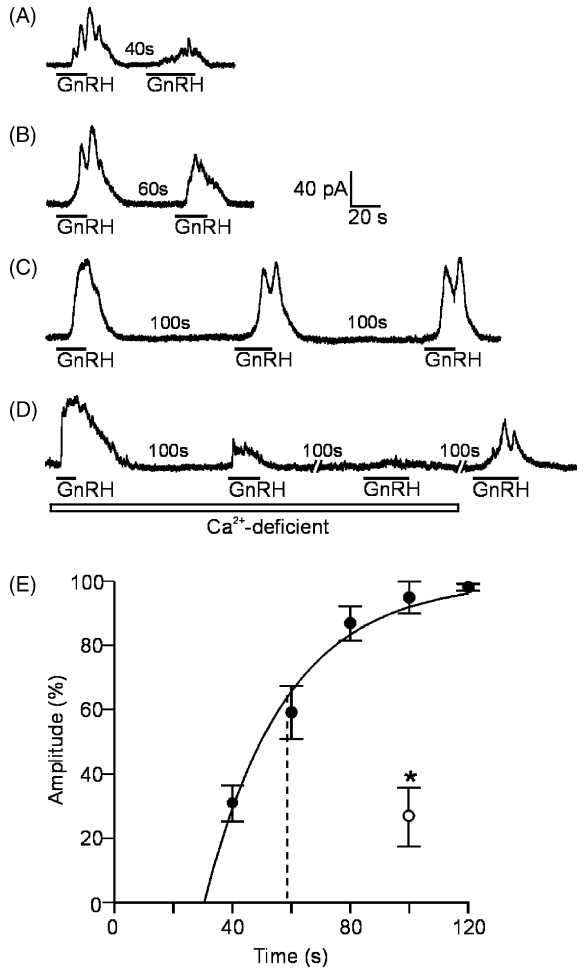

Fig. 4. Recovery of GnRH-induced monophasic calcium response. (A–C) Effects of variable interpulse intervals on recovery of monophasic calcium signals. (D) Effects of removal of extracellular calcium on recovery of agonist-induced calcium signals. Cells were stimulated with 10 nM GnRH. The current traces shown in panels A to D are from the same cell maintained at −40 mV. (E) The amplitude of the second GnRH response expressed in percentage of control GnRH-induced current and plotted as a function of time interval between two GnRH pulses. Data from individual cells were fitted to a single exponential equation. The theoretical curve started at ∼30 s, which corresponds to the refractory period when no response could be evoked. Starting from this time point, the response recovered with $\tau = 28.3 \pm 8.2$ s ($n = 4$). Dotted vertical line indicates the time corresponding to calculated τ value. Each point represents the mean ± S.E.M. from three to five cells recorded in calcium-containing (filled circles) and calcium-deficient solution (open circle).

second and subsequent responses progressively decreased (Fig. 6B), and with reintroduction of calcium the recovery of response was observed.

3.5. Effect of sodium-deficient medium on GnRH-induced calcium responses

In general, calcium efflux from the cells occurs through plasma membrane calcium ATPase and/or Na^+/Ca^{2+} exchanger [27]. To test the relevance of extracellular sodium on Ca^{2+} efflux, cells were initially stimulated with sodium-

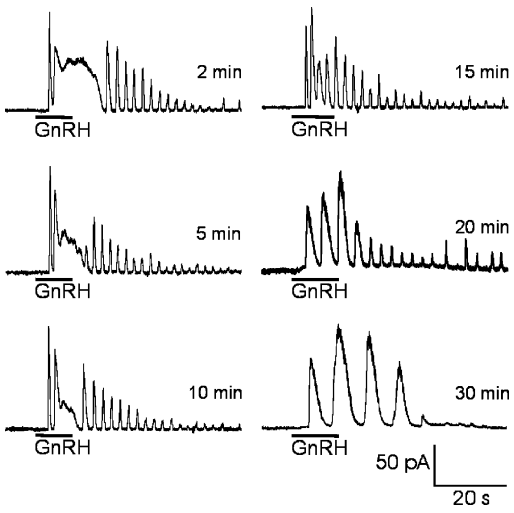

Fig. 5. Changes in the shape of GnRH-induced calcium signals during prolonged patch-clamp recording from a single cell. The biphasic responses were induced by repetitive stimulation of a cell with 10 nM GnRH. No obvious changes in the pattern of signaling was observed during first four GnRH pulses (initiated 2, 5, 10, and 15 min after the initial GnRH stimulation). Further stimulation led to increase in duration of single transients, whereas the baseline pattern was preserved (20 and 30 min).

containing medium, followed by stimulation in the presence of NMDG and the third GnRH pulse was again applied in the presence of sodium. Three types of signals, the rapid baseline oscillations (Fig. 7A), biphasic response (Fig. 7B), and slow baseline oscillations (Fig. 7C) were analyzed. As shown in Fig. 7A, the frequency of GnRH-induced rapid baseline oscillations did not change in Na^+-deficient solution (the duration of individual spike was 2.64 ± 0.17 s control and 2.76 ± 0.187 s in NMDG-containing medium; $n = 6$; Fig. 7D). However, the duration of oscillations was prolonged, in average for about $36 \pm 12\%$ (Fig. 7A, middle panel). The duration of the initial non-oscillatory spike of biphasic response was substantially prolonged, in average more than two times (from 32.9 ± 6.2 s to 69.2 ± 9.4 s; $n = 5$; $P < 0.01$) when cells were perfused with NMDG-containing medium (Fig. 7B and E). Also, the duration of individual spikes of GnRH-induced low-frequency baseline oscillations was significantly prolonged (from 7.0 ± 0.7 s to 11.8 ± 1.4 s;

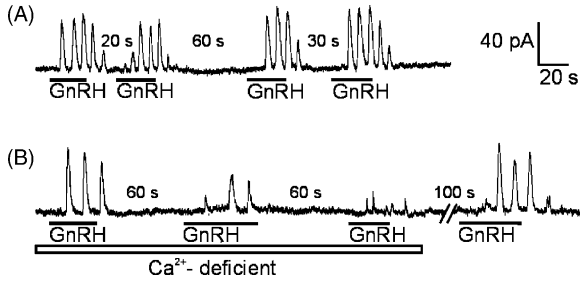

Fig. 6. Extracellular calcium-dependence of GnRH-induced slow baseline calcium oscillations. (A) Pattern of calcium signaling in cells perfused with calcium-containing medium. (B) Effects of removal of extracellular calcium on recovery of calcium signals.

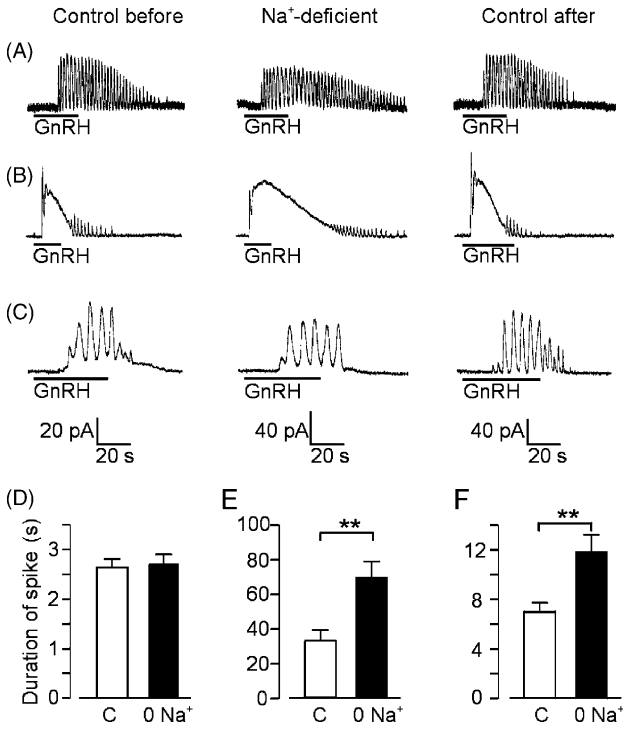

Fig. 7. Effects of extracellular sodium on time-course of calcium signaling. (A) Rapid baseline oscillations; (B) biphasic response; (C) slow baseline oscillations. In each panel, the current traces shown from left to right are from the same cell. All cells were stimulated with 10 nM GnRH in sodium-containing medium (the first and the third trace) and sodium-deficient and NMDG-containing medium (the second trace). (D–F) Averaged duration of individual calcium spikes in sodium-containing (open bars) and sodium-deficient solution (filled bars); (D) rapid baseline oscillations; (E) biphasic and monophasic response; (F) slow baseline oscillations. Asterisks (**) indicate $P < 0.01$ vs. sodium-containing medium.

$n = 4$; $P < 0.01$) in NMDG-perfused cells (Fig. 7C and F). These results parallel the findings with extracellular calcium-deficient medium, confirming the hypothesis that the efficacy of two systems for handling cytosolic calcium is determined by the pattern of calcium mobilization.

4. Discussion

The principal problem in studies with repetitive stimulation of cells with calcium-mobilizing agonist is what underlies the kinetics of recovery of calcium signaling: the receptor desensitization–internalization–resensitization cycle, the washout effect, and/or the depletion–repletion kinetics of agonist-sensitive calcium pool. Unlike other G protein-coupled receptors, GnRH receptor does not desensitize [28]. This is due to absence of C-terminal tail in this receptor [29], which is responsible for desensitization of non-mammalian GnRH receptors [30], as well as other mammalian calcium-mobilizing receptors [31,32]. The reservoir of PIP_2 for repetitive activation of this receptor is also unlikely to represents the limiting factor in gonadotrophs, because only during the prolonged stimulation (over 20 min) such depletion starts to occur [33], and in our experiments cells were perfused with GnRH only for 10–30 s.

In neonatal gonadotrophs, the estimated EC_{50} for GnRH-induced current response was 2.4 nM and the maximal effect was observed at 100 nM [6]. In postnatal gonadotrophs, perfused at slower rate than in our patch-clamp experiments, the washout half-time of ^{125}I-GnRH was around 25 s [34]. In this study, we used usually 10 nM GnRH and we assume that a majority of receptors become available during the washout time, as well as that additional receptors could be activated during repetitive GnRH applications. Consistent with this hypothesis, GnRH II, a GnRH receptor agonist that has about 50 times lower affinity for receptors in neonatal gonadotrophs, induced current responses that recovered with similar time constant as responses induced by GnRH (Zemkova, unpublished observation). Thus, the recovery of IP_3-sensitive calcium pool in neonatal gonadotrophs should predominantly depend on redistribution of calcium within the intracellular calcium pools and its efflux/influx through plasma membrane.

The major finding in our study is that two principal patterns of GnRH-induced calcium signals, rapid baseline oscillations and monophasic/biphasic responses, exhibit different sensitivity to extracellular calcium and sodium. Rapid baseline calcium oscillations were only partially influenced by removal of extracellular calcium during repetitive GnRH stimulation. In contrast, in cells responding with monophasic/biphasic signaling and slow baseline oscillations, stimulation with GnRH for 20–30 s in Ca^{2+}-deficient medium was sufficient to abolish the subsequent response to this agonist. Similarly, removal of extarcellular sodium had only a minor effect on duration of rapid baseline oscillations, but significantly prolonged the duration of spike response. These results indicate that in cells responding with rapid baseline oscillations the majority of calcium is recycled within the cells, whereas in cells responding with monophasic/biphasic signals and slow oscillations the majority of Ca^{2+} was removed from the cells, presumably by activation of Na^+/Ca^{2+} exchanger. Thus, the efficacy of Na^+-dependent Ca^{2+} efflux mechanism is influenced by the pattern of calcium signaling. The sensitivity of Na^+/Ca^{2+} exchange to cytosolic calcium is lower but the transport rate of this system is at least 30 times higher than that controlled by (Ca^{2+})ATPase in the intracellular stores, which functions at or near its maximal rate at resting $[Ca^{2+}]_i$ [35].

Our findings are in general agreement with Hille's data in postnatal gonadotrophs from male rats [36] showing the relevance of rapid calcium uptake by mitochondria and its gradual release from mitochondria. We may speculate that such redistribution of calcium within the neonatal gonadotrophs enables these cells to respond to repetitive stimulation with GnRH when bathed in calcium-deficient medium. In that scenario, the redistribution of calcium within the cells is favored by rapid baseline oscillations, whereas activation of Na^+/Ca^{2+} exchanger requires a prolonged exposure to

elevated $[Ca^{2+}]_i$, as it is observed in slow baseline oscillations and spike response. To this end, it is important to stress that current measurements compared to calcium measurements provide better estimate of calcium concentration in the plasma membrane subdomain [5] that is of the relevance for efficacy of sodium-dependent efflux mechanism.

Furthermore, we observed a consistent difference in the kinetics of recovery of rapid baseline oscillations versus monophasic/biphasic signals. First, there is no a refractory period in recovery of rapid baseline oscillations, whereas monophasic/biphasic response had a 30-s long refractory period. Second, the recovery time constant for rapid baseline oscillations was about 20 s, and for the spike response was close to 30 s. Because the recovery of baseline oscillations, but not monophasic/biphasic response, was practically unaffected by removal of extracellular calcium, these observations suggest that in our experimental conditions the refilling of $InsP_3$-sensitive calcium pool from intracellular stores is faster process than from calcium influx from extracellular medium.

Our experiments do not clarify through which channels GnRH-induced calcium influx supplements calcium mobilization in neonatal gonadotrophs. Postnatal gonadotrophs express voltage-gated calcium channels [37], and their activation is essential for calcium signaling during sustained agonist stimulation [38]. At the present time, it is unknown whether voltage-gated calcium channels are also expressed in neonatal gonadotrophs. Here, all experiments were done in cells clamped at −40 mV, a protocol that does not completely eliminate voltage-gated Ca^{2+} entry in cells expressing these channels. Furthermore, this protocol modulates Ca^{2+} entry caused by the natural oscillatory membrane potential changes into steady-state Ca^{2+} influx. These experiments also do not exclude the participation of voltage-insensitive capacitative calcium entry through store-operated channels [39], if operative in these cells. All together, further experiments are needed to clarify the nature of calcium influx in these cells.

Although it is generally accepted in the field that Na^+/Ca^{2+} exchange plays a role in control of calcium signaling [27], and this pathway is integrated in theoretical model of GnRH-induced calcium oscillations in gonadotrophs [21], there are no experimental data to support this hypothesis. We believe there is only one publication addressing the potential role of extracellular sodium on GnRH-induced secretion in postnatal gonadotrophs [40]. In this study, removal of sodium attenuated GnRH-induced gonadotropin secretion during the prolonged (3 h) agonist stimulation. Further experiments are needed to clarify the operation of Na^+/Ca^{2+} exchange system in postnatal gonadotrophs and the impact of blockade of sodium-dependent calcium efflux pathway on rapid gonadotropin secretion in neonatal and postnatal gonadotrophs.

Finally, we observed that the latency time differs for the different response patterns. During the lag phase, activated GnRH receptor stimulates phospholipase C, intracellular $InsP_3$ is generated and after reaching threshold level, Ca^{2+} channel coupled to $InsP_3$ receptor is activated. The duration of latency is thus inversely related to the concentration of agonist and intracellular $InsP_3$. In a subpopulation of melatonin-sensitive gonadotrophs, the latency also dependents on the presence of extracellular Ca^{2+} [6], indicating that extracellular Ca^{2+} entry helps to initiate Ca^{2+} release from $InsP_3$-sensitive stores. Another question is variability of latency time between different types of GnRH-induced responses, which does not correlate with extracellular Ca^{2+} sensitivity [6]. We may speculate that some unknown agonist-stimulated intracellular factor is generated, which has stimulatory or inhibitory affect on the cascade of events preceding the initiation of $InsP_3$-induced Ca^{2+} release. Another possibility is that the duration of latency reflects specific structural properties, which could vary from cell to cell and influence the initiation and the pattern of signaling.

In summary, our data provide novel information about the possible influence of the pattern of signaling on calcium handling by the cells. It appears that activation of rapid baseline oscillations limits the efficacy of low-sensitive sodium-dependent calcium efflux pathway. This in turn makes cells less dependent on calcium influx and redistribution of calcium within the cells provides more rapid recovery of $InsP_3$-sensitive calcium pool. A significant influence of sodium-dependent calcium efflux pathway on monophasic/biphasic responses and slow oscillations could be of the physiological relevance, i.e. to reduce the harmful effect of prolonged elevation in $[Ca^{2+}]_i$ for cells.

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References

[1] E. Seuntjens, A. Hauspie, H. Vankelecom, C. Denef, Ontogeny of plurihormonal cells in the anterior pituitary of the mouse, as studied by means of hormone mRNA detection in single cells, J. Neuroendocrinol. 14 (2002) 611–619.
[2] M. Tsutsumi, W. Zhou, R.P. Millar, P.L. Mellon, J.L. Roberts, C.A. Flanagan, K. Dong, B. Gillo, S.C. Sealfon, Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor, Mol. Endocrinol. 6 (1992) 1163–1169.
[3] J. Reinhart, L.M. Mertz, K.J. Catt, Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor, J. Biol. Chem. 267 (1992) 21281–21284.
[4] A. Tse, B. Hille, GnRH-induced Ca^{2+} oscillations and rhythmic hyperpolarizations of pituitary gonadotropes, Science 255 (1992) 462–464.
[5] M. Kukuljan, S.S. Stojilkovic, E. Rojas, K.J. Catt, Apamin-sensitive potassium channels mediate agonist-induced oscillations of

membrane potential in pituitary gonadotrophs, FEBS Lett. 301 (1992) 19–22.
[6] H. Zemkova, J. Vanecek, Differences in gonadotropin-releasing hormone-induced calcium signaling between melatonin-sensitive and melatonin-insensitive neonatal rat gonadotrophs, Endocrinology 141 (2000) 1017–1026.
[7] J. Vanecek, The melatonin receptors in rat ontogenesis, Neuroendocrinology 48 (1988) 201–203.
[8] J. Vanecek, D.C. Klein, Melatonin inhibits gonadotropin-releasing hormone-induced elevation of intracellular Ca^{2+} in neonatal rat pituitary cells, Endocrinology 130 (1992) 701–707.
[9] O.H. Petersen, C.C. Petersen, H. Kasai, Calcium and hormone action, Annu. Rev. Physiol. 56 (1994) 297–319.
[10] K. Kiselyov, D.M. Shin, S. Muallem, Signalling specificity in GPCR-dependent Ca^{2+} signalling, Cell Signal. 15 (2003) 243–253.
[11] G.A. Shangold, S.N. Murphy, R.J. Miller, Gonadotropin-releasing hormone-induced Ca^{2+} transients in single identified gonadotropes require both intracellular Ca^{2+} mobilization and Ca^{2+} influx, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 6566–6570.
[12] S.S. Stojilkovic, T. Iida, F. Merelli, K.J. Catt, Calcium signaling and secretory responses in endothelin-stimulated anterior pituitary cells, Mol. Pharmacol. 39 (1991) 762–770.
[13] D.A. Leong, M.O. Thorner, A potential code of luteinizing hormone-releasing hormone-induced calcium ion responses in the regulation of luteinizing hormone secretion among individual gonadotropes, J. Biol. Chem. 266 (1991) 9016–9022.
[14] T. Iida, S.S. Stojilkovic, S. Izumi, K.J. Catt, Spontaneous and agonist-induced calcium oscillations in pituitary gonadotrophs, Mol. Endocrinol. 5 (1991) 949–958.
[15] S.S. Stojilkovic, M. Kukuljan, M. Tomic, E. Rojas, K.J. Catt, Mechanism of agonist-induced $[Ca^{2+}]_i$ oscillations in pituitary gonadotrophs, J. Biol. Chem. 268 (1993) 7713–7720.
[16] M. Tomic, M. Cesnjaj, K.J. Catt, S.S. Stojilkovic, Developmental and physiological aspects of Ca^{2+} signaling in agonist-stimulated pituitary gonadotrophs, Endocrinology 135 (1994) 1762–1771.
[17] F.W. Tse, A. Tse, B. Hille, H. Horstmann, W. Almers, Local Ca^{2+} release from internal stores controls exocytosis in pituitary gonadotrophs, Neuron 18 (1997) 121–132.
[18] F. Merelli, S.S. Stojilkovic, T. Iida, L.Z. Krsmanovic, L. Zheng, P.L. Mellon, K.J. Catt, Gonadotropin-releasing hormone-induced calcium signaling in clonal pituitary gonadotrophs, Endocrinology 131 (1992) 925–932.
[19] I. Bezprozvanny, J. Watras, B.E. Ehrlich, Bell-shaped calcium-response curves of $Ins(1,4,5)P_3$- and calcium-gated channels from endoplasmic reticulum of cerebellum, Nature 351 (1991) 751–754.
[20] G.W. De Young, J. Keizer, A single-pool inositol 1,4,5-trisphosphate-receptor-based model for agonist-stimulated oscillations in Ca^{2+} concentration, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 9895–9899.
[21] Y.X. Li, J. Rinzel, J. Keizer, S.S. Stojilkovic, Calcium oscillations in pituitary gonadotrophs: comparison of experiment and theory, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 58–62.
[22] S.S. Stojilkovic, M. Tomic, M. Kukuljan, K.J. Catt, Control of calcium spiking frequency in pituitary gonadotrophs by a single-pool cytoplasmic oscillator, Mol. Pharmacol. 45 (1994) 1013–1021.
[23] J.W. Putney Jr., G.S. Bird, The inositol phosphate-calcium signaling system in nonexcitable cells, Endocr. Rev. 14 (1993) 610–631.
[24] R.N. Mandler, A.E. Schaffner, E.A. Novotny, G.D. Lange, S.V. Smith, J.L. Barker, Electrical and chemical excitability appear one week before birth in the embryonic rat spinal cord, Brain Res. 522 (1990) 46–54.
[25] J. Vanecek, D.C. Klein, A subpopulation of neonatal gonadotropin-releasing hormone-sensitive pituitary cells is responsive to melatonin, Endocrinology 133 (1993) 360–367.
[26] H. Zemkova, J. Vanecek, Inhibitory effect of melatonin on gonadotropin-releasing hormone-induced Ca^{2+} oscillations in pituitary cells of newborn rats, Neuroendocrinology 65 (1997) 276–283.
[27] M.J. Berridge, Inositol trisphosphate and calcium signalling, Nature 361 (1993) 315–325.
[28] J.N. Hislop, M.T. Madziva, H.M. Everest, T. Harding, J.B. Uney, G.B. Willars, R.P. Millar, B.E. Troskie, J.S. Davidson, C.A. McArdle, Desensitization and internalization of human and Xenopus gonadotropin-releasing hormone receptors expressed in alphaT4 pituitary cells using recombinant adenovirus, Endocrinology 141 (2000) 4564–4575.
[29] C.A. McArdle, J.S. Davidson, G.B. Willars, The tail of the gonadotrophin-releasing hormone receptor: desensitization, at and distal to, G protein-coupled receptors, Mol. Cell. Endocrinol 151 (1999) 129–136.
[30] C. Tensen, K. Okuzawa, M. Blomenrohr, F. Rebers, R. Leurs, J. Bogerd, R. Schulz, H. Goos, Distinct efficacies for two endogenous ligands on a single cognate gonadoliberin receptor, Eur. J. Biochem. 243 (1997) 134–140.
[31] R. Yu, P.M. Hinkle, Desensitization of thyrotropin-releasing hormone receptor-mediated responses involves multiple steps, J. Biol. Chem. 272 (1997) 28301–28307.
[32] J.A. Olivares-Reyes, R.D. Smith, L. Hunyady, B.H. Shah, K.J. Catt, Agonist-induced signaling, desensitization, and internalization of a phosphorylation-deficient AT1A angiotensin receptor, J. Biol. Chem. 276 (2001) 37761–37768.
[33] C.A. McArdle, W. Forrest-Owen, G. Willars, J. Davidson, A. Poch, M. Kratzmeier, Desensitization of gonadotropin-releasing hormone action in the gonadotrope-derived alpha T3-1 cell line, Endocrinology 136 (1995) 4864–4871.
[34] S.S. Stojilkovic, A. Stutzin, S.-I. Izumi, S. Dufour, A. Torsello, M.A. Virmani, E. Rojas, K.J. Catt, Generation and amplification of the cytosolic calcium signal during secretory responses to gonadotropin-releasing hormone, New Biol. 2 (1990) 272–283.
[35] B.J. Baum, I.S. Ambudkar, V.J. Horn, Evidence that ATP-dependent Ca^{2+} transport in rat parotid microsomal membranes requires charge compensation, Biochem. J. 254 (1988) 649–654.
[36] E.J. Kaftan, T. Xu, R.F. Abercrombie, B. Hille, Mitochondria shape hormonally induced cytoplasmic calcium oscillations and modulate exocytosis, J. Biol. Chem. 275 (2000) 25465–25470.
[37] A. Stutzin, S.S. Stojilkovic, K.J. Catt, E. Rojas, Characteristics of two types of calcium channels in rat pituitary gonadotrophs, Am. J. Physiol. 257 (1989) C865–C874.
[38] S.S. Stojilkovic, M. Kukuljan, T. Iida, E. Rojas, K.J. Catt, Integration of cytoplasmic calcium and membrane potential oscillations maintains calcium signaling in pituitary gonadotrophs, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 4081–4085.
[39] J.W. Putney Jr., R.R. McKay, Capacitative calcium entry channels, Bioessays 21 (1999) 38–46.
[40] C.A. McArdle, E.J. Cragoe Jr., A. Poch, Na^+ dependence of gonadotropin-releasing hormone action: characterization of the Na^+/H^+ antiport in pituitary gonadotropes, Endocrinology 128 (1991) 771–778.